THE EFFECT OF OLANZAPINE ON THE SYNAPTIC TRANSMISSION OF THE DORSAL MOTOR NUCLEUS OF THE VAGUS

AN ABSTRACT

SUBMITTED ON THE TENTH DAY OF APRIL 2014

TO THE DEPARTMENT OF NEUROSCIENCE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF SCIENCE AND ENGINEERING

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

MASTER OF SCIENCE IN NEUROSCIENCE

BY

IMRAN JOHN ANWAR

APPROVED:

Andrei V. Derbenev, Ph.D.

Andrea Zsombok, Ph.D.

Jeffrey G. Tasker, Ph.D.

ABSTRACT

Olanzapine, an atypical antipsychotic, alleviates symptoms of schizophrenia while producing fewer side effects compared to first generation antipsychotics. However, chronic usage remains problematic due to the propensity of olanzapine to induce weight gain and metabolic disturbances. Moreover, the cellular mechanisms underlying the metabolic side effects are poorly understood. The central nervous system (CNS) exerts both hormonal and neuronal control over whole body homeostasis. The dorsal motor nucleus of the vagus (DMV) participates in this regulation through modulation of the parasympathetic outflow to subdiaphragmatic organs. We hypothesized that olanzapine disrupts neurotransmission of the DMV, and thus contributes to the dysregulation of metabolism. We used whole-cell patch-clamp recordings from female C57BL/6J to assess the effect of olanzapine on DMV neurons. First, we investigated the effect of acute olanzapine administration on the activity of DMV neurons. Acute application of 10 μM olanzapine on DMV neurons induced both pre- and postsynaptic effects. Voltage-clamp recordings revealed that, in 5 out of 9 DMV neurons, excitatory inputs to DMV neurons were significantly increased by 71.6 ± 22.1%. In addition, in current-clamp mode, olanzapine induced a robust hyperpolarization from -49.00 ± 0.64 mV to -60.82 ± 2.78 mV. The hyperpolarization suppressed action potential firing. As a next step, we investigated the subchronic effect of olanzapine on the activity of DMV neurons. Daily subcutaneous injections were made for 20 days (5 mg/kg/day of olanzapine and vehicle). We did not find significant differences in body weight, blood glucose, and insulin or leptin levels. Subchronic administration of olanzapine generated presynaptic changes in DMV neurons. In treated animals, additional infusion of 10 μM olanzapine on DMV neurons significantly reduced excitatory neurotransmission by 41.0 ± 3.1% in 10 out of 17 neurons. Our findings indicate that olanzapine directly modulates the neuronal activity in DMV neurons, and could thus contribute to the metabolic disturbances seen in long-term treatments.

EFFECT OF OLANZAPINE ON THE SYNAPTIC TRANSMISSION OF THE DORSAL MOTOR NUCLEUS OF THE VAGUS

A THESIS

SUBMITTED ON THE TENTH DAY OF APRIL 2014

TO THE DEPARTMENT OF NEUROSCIENCE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF SCIENCE AND ENGINEERING

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

MASTER OF SCIENCE IN NEUROSCIENCE

BY

IMRAN JOHN ANWAR

APPROVED:

Andrei V. Derbenev, Ph.D.

Andrea Zsombok, Ph.D.

Jeffrey G. Tasker, Ph.D.

ACKNOWLEDGMENT

This work is supported by the NIH P30GM193337 and the Tulane SOM pilot Project. I would like to thank Drs. Andrea Zsombok, Kayoko Myata, Hong Gao, Andrei Derbenev and Jeffery Tasker for all of their invaluable help and support. In particular, I would like to thank Drs. Andrea Zsombok, Andrei Derbenev, and Jeffrey Tasker for the accepting to be on my thesis committee and for providing invaluable feedbacks on my project.

TABLE OF CONTENTS

ABBREVIATIONS

5-HT: 5-hydroxytryptamine

aCSF: artificial cerebrospinal fluid

ANS: autonomic nervous system

AP: action potential

CB1R: type 1 cannabinoid receptor

CNS: central nervous system

DMV: dorsal motor nucleus of vagus nerve

DVC: dorsal vagal complex

EPSC: excitatory postsynaptic current

mEPSC: miniature excitatory postsynaptic current

NTS: nucleus of the solitary tract

PNS: peripheral nervous system

PSC: postsynaptic current

RMP: resting membrane potential

sEPSC: spontaneous excitatory postsynaptic current

TTX: tetrodotoxin

INTRODUCTION

Olanzapine alleviates both positive (e.g. hallucinations and delusions) and negative symptoms of schizophrenia (e.g. anhedonia and flat affect) while producing fewer side effects compared to first-generation antipsychotics (Beasley et al., 1996; Leucht et al., 1999). As an atypical antipsychotic, olanzapine possesses a favorable clinical application due to its higher affinity for serotonin (5-hydroxytryptamine; 5-HT) 5-HT_{2A} receptor then to dopamine receptor D_2, significantly reducing the extrapyramidal symptoms mediated by the dopamine system (Bymaster et al., 1996; Zhang and Bymaster, 1999). Additionally, olanzapine has high affinity for other receptors: *in vitro*, it is an antagonist for dopamine (D_1, D4), serotonin 5-$HT_{2c,}$ adrenergic (α_1), histamine (H_1), and muscarinic receptors (Fulton and Goa, 1997; Moore et al., 1992). However, due to its complex pharmacodynamic profile, olanzapine therapy results in a variety of adverse effects that are not completely understood.

Indeed, chronic usage remains problematic due to the propensity of olanzapine to induce metabolic disturbances, such as weight gain and impaired blood glucose regulation (Allison et al., 1999; American Diabetes Association et al., 2004; Beasley et al., 1996; Fulton and Goa, 1997; Ratzoni et al., 2002). Various lines of evidence suggest that chronic olanzapine treatment induces metabolic disturbances through olanzapine's hyperphagic effects, as shown both in humans and in animal models (Blouin et al., 2012; Cooper et al., 2005). The cellular mechanisms underlying the metabolic side effects remain however poorly understood.

The central nervous system (CNS) exerts control on whole-body homeostatic processes, such as energy homeostasis and blood glucose regulation, via regulation of the autonomic nervous system (Carnethon et al., 2003; Morton et al., 2006; Sandoval et al., 2008; Zsombok and Smith, 2009). The dorsal vagal complex (DVC), a group of nuclei in the caudal dorsomedial medulla oblongata, participates in this regulation though modulation of the vagal nerve output (Carnethon et al., 2003; Marino et al., 2011; Mussa and Verberne, 2013; Zsombok and Smith, 2009; Zsombok et al., 2011a).

The DVC encompasses the nucleus of the solitary tract (NTS), the dorsal motor nucleus of the vagus nerve (DMV), and the area postrema (AP). The NTS receives inputs from cranial visceral afferents that carry viscerosensory information as well as inputs from other brain areas such as the hypothalamus

(Morton et al., 2006). The interneurons in the NTS then send outputs to various targets, including the DMV (Davis et al., 2003; Glatzer and Smith, 2005; Travagli and Rogers, 2001; Travagli et al., 1991). The DMV finally sends vagal projections to postganglionic neurons innervating subdiaphragmatic organs (Browning and Travagli, 2011), thereby regulating the function of the gastrointestinal tract, the cardiovascular system, and the respiratory system (Bauer et al., 2005; Cavanaugh et al., 2011; Shoudai et al., 2010; Zsombok et al., 2011b). Together, these nuclei are critical for the correct functioning of various organs and consequently of various whole-body homeostatic processes.

In particular, it is well known that the DVC has a central role in energy homeostasis, where it integrates signals relevant to both food intake and body weight regulation (Berthoud, 2002). The NTS receives direct information about taste, esophageal distension, and stomach distension through vagal primary afferents (Cunningham and Sawchenko, 1989; Deutsch, 1985; Roussin et al., 2012). In addition to direct neuronal projections, the DVC also receives information about energy homeostasis through both orexigenic and anorexigenic hormones, such as melanocortin-4, ghrelin, nedtastin-1, and leptin (Grill et al., 2002; Williams et al., 2000; 2006; Zhang et al., 2013). The activity of the DVC is tightly regulated by both neuronal and hormonal signals related to energy homeostasis.

While it is well known that the DVC is involved in energy homeostasis and that olanzapine alters energy homeostasis, relatively little is known about the interaction of olanzapine and DVC. Recently, it has been shown that chronic olanzapine administration reduces type 1 cannabinoid receptors (CB1R) binding density in the DVC (Weston-Green et al., 2012; 2008). CB1R activation has been shown to inhibit synaptic inputs to the DMV (Derbenev et al., 2004); The reduction of CB1R binding density could counteract the tonic inhibition exerted by the cannabinoid system on the vagal output. We then hypothesized that olanzapine disrupts neurotransmission of the DMV, and thus contributes to the dysregulation of metabolism.

We first investigated if acute administration of olanzapine could modify the neuronal activity of DMV neurons. We show that acute application of olanzapine increases spontaneous excitatory synaptic inputs to the DMV and reduces the neuronal activity of DMV neurons. We then looked at the neuroplasticity occurring in the DVC resulting from subchronic olanzapine administration. In olanzapine-treated animals, the neuronal activity of DMV neurons did not differ significantly from control. We show

that subchronic olanzapine treatment changes the olanzapine-dependent regulation of excitatory inputs: in olanzapine-treated animals, acute olanzapine administration reduces excitatory inputs to DMV neurons. Finally, we show that the olanzapine-dependent hyperpolarization of DMV neurons is still present in olanzapine-treated animals. This demonstration of olanzapine-dependent regulation of DMV neurons suggests that olanzapine alters autonomic circuitry, possibly contributing to weight gain and hyperglycemia by decreasing the excitability of DMV neurons and reducing vagal output.

RESEARCH DESIGN AND METHODS

Experiments were performed on female C57BL/6 mice (5-14 weeks old; Harlan) following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Tulane University's Institutional Animal Care and Use Committee.

Injection protocol

Olanzapine (Tocris Bioscience) was dissolved in 0.1 M citric acid and added to 0.9% sterile saline. The pH was then adjusted to 5.5 with addition of 1 M NaOH. Vehicle solution consisted of 0.9% sterile saline with the same amount of citric acid utilized to dissolve olanzapine. The pH was also adjusted to 5.5.

Female C57BL/6J mice were randomly assigned to receive either olanzapine treatment (5 mg/kg/day) or vehicle. Daily subcutaneous injections of either olanzapine or vehicle were made depending on their group. The solutions were made daily before each administration.

Brainstem slice preparation

Transverse brainstem slices were prepared from female C57BL/6J mice (5-14 weeks old; Harlan) as described previously (Zsombok et al., 2011a). Briefly, mice were deeply anesthetized by isoflurane inhalation and sacrificed by decapitation while anesthetized. Brains were rapidly removed and immersed in ice-cold (0 – 4°C) oxygenated (95%O_2 - 5%CO_2) artificial CSF (aCSF) containing the following: 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.4 mM NaH_2PO_4, 11 mM glucose, 1.3 mM $CaCl_2$, and, 1.3 mM $MgCl_2$. The pH was adjusted to physiological ranges (7.2–7.4), with an osmolality of 290–310 mOsm/kg. Transverse brainstem slices (300 μm) containing the DMV were made using a vibrating microtome (Vibratome Series 1000; Technical Products, St. Louis, MO). Slices were maintained in an oxygenated bath at 32°C for at least one hour before performing experiments. Slices were then transferred to a recording chamber mounted on a fixed stage under an upright microscope (Gao et al., 2012).

Whole-cell patch-clamp recordings

DMV neurons were visually identified in coronal brainstem slices and were patch-clamped with a glass pipette typically having a series resistance between 2 to 4 MΩ. The electrodes were filled with a solution containing the following: 130 mM Cs^+-gluconate, 1 mM NaCl, 5 mM EGTA, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM CsOH, 2-4 mM Mg-ATP, and 0.2% biocytin. The pH was then adjusted to

physiological ranges (7.2-7.4) with 5M CSOH. Some of the recordings were done using 130 mM K^+-gluconate instead of Cs^+-gluconate. Electrophysiological signals were recorded using an Axoclamp 700B amplifier (Molecular Devices) and acquired by pClamp (Molecular Devices). Excitatory postsynaptic currents (EPSCs) were examined at a holding potential of -60 mV. Action potentials (AP) were examined at a holding current of 0 pA, unless specified otherwise.

Drug application

Some of the recordings were performed with tetrodotoxin (1 μM; TTX; Tocris Bioscience) in aCSF to block action potentials. In addition, olanzapine (10 μmol/L; Tocris Bioscience) and the CB_1 antagonist AM251 (10 μmol/L; Tocris Bioscience) were dissolved in DMSO and diluted in aCSF (final concentration of DMSO <0.01% by volume).

Data analysis

The recordings were analyzed with pClamp 10 software (Molecular Devices). Spontaneous and miniature IPSCs and EPSCs were analyzed offline using MiniAnalysis (Synaptosoft). The effects of olanzapine on EPSCs and IPSCs were analyzed within individual cells using the Kolmogorov-Smirnov test. The effects of olanzapine across neuron groups were analyzed using a paired two-tailed Student's *t* test or between different groups using one-way ANOVA. Values are expressed as means ± SEM.

RESULTS

Olanzapine potentiates excitatory inputs to DMV neurons in an AP-dependent manner.

To investigate excitatory synaptic regulation of DMV neurons by acute administration of olanzapine, we used whole-cell patch-clamp recordings. The frequency of excitatory postsynaptic currents (EPSCs) was examined at -60 mV using K^+-gluconate solution in the recording pipette.

Bath application of olanzapine (10 μM) significantly increased the overall frequency of sEPSCs (Fig. 1). The effect of olanzapine was observed within 3 minutes of reaching the slice and reached a maximal effect after 5 minutes. In control conditions, the average sEPSC frequency was 2.30 ± 0.52 Hz (range 0.93-6.32 Hz). After 5 minutes of olanzapine infusion, the average sEPSC frequency was increased to 3.23 ± 0.55 Hz (range 1.87-7.08 Hz; n = 9; $P < 0.01$) (Fig. 1A, 1B). Additional analysis with Kolmogorov-Smirnov test revealed that 6 out of 9 cells has a significant increase in sEPSC frequency, indicating that not all DMV neurons are responsive to olanzapine (Fig. 1C, 1D).

To investigate whether this increase of sEPSC frequency is mediated by AP-independent mechanisms, recordings were made in the presence of TTX (1 μM) to look at miniature EPSCs (mEPSCs). In TTX, the average mEPSC frequency was 1.54 ± 0.21 Hz (range 0.59-2.51 Hz). Co-application of TTX and olanzapine failed to elicit an increase in the average mEPSC frequency (1.48 ± 0.212 Hz; range 0.43-2.32; n = 10; $P > 0.05$) (Fig. 1B). This suggests that olanzapine potentiates excitatory synaptic inputs to DMV neurons in an AP-dependent manner.

Olanzapine reduces the neuronal excitability of DMV neurons in an AP-independent fashion.

Next, we looked at the overall effect of olanzapine on the neuronal excitability of DMV neurons. Bath application of olanzapine reduced the firing of action potentials (APs) of DMV neurons (Fig. 2). In current-clamp mode, all DMV neurons were spontaneously firing APs. The average AP frequency was 2.90 ± 0.69 Hz (range 0.53-8.47 Hz). In olanzapine, the average AP frequency was significantly reduced to 0.23 ± 0.17 Hz (range 0-1.76 Hz; n = 10; $P < 0.01$) (Fig. 2A, 2C). However, olanzapine failed to elicit a change in AP threshold compared to control conditions. In control conditions, AP threshold was -39.72 ± 1.22 mV

(range -37.02- -42.76 mV) compared to -40.70 ± 1.61 mV when olanzapine was present in the bath (range -37.47- -45.06 mV; n = 4; $P > 0.05$) (Fig. 2D).

Additionally, olanzapine induced a large hyperpolarization of DMV neurons. In control conditions, the average resting membrane potential (RMP) was -49.00 ± 0.63 mV (range -44.84- -51.15 mV). Application of olanzapine hyperpolarized DMV neurons to -60.27 ± 2.78 mV (range -49.38- -70.73 mV; n = 9; $P < 0.01$), resulting in a net change of RMP of -11.28 ± 2.70 mV (range -20.87- -0.99 mV) (Fig. 2E).

To investigate whether the olanzapine-induced decrease of neuronal excitability is mediated by a postsynaptic mechanism, recordings were made in the presence of TTX to block APs. In TTX, the average RMP was -42.12 ± 1.97 mV (range -29.80- -53.16 mV; n = 10). Co-application of TTX and olanzapine hyperpolarized DMV neurons to -57.63 ± 3.42 mV (range -40.99- -71.93 mV; n = 10; $P < 0.01$), resulting in a net change of RMP of -15.51 ± 4.04 mV (Fig. 2B, 2E). The olanzapine-induced hyperpolarization in TTX (-15.51 ± 4.04 mV) was similar then in control conditions (-11.28 ± 2.70 mV; $P > 0.05$). Taken together, these results suggest that olanzapine reduces the neuronal excitability of DMV neurons by hyperpolarizing them in an AP-independent mechanism.

Subchronic olanzapine treatment does not affect metabolic characteristics.

We performed daily subcutaneous injections of olanzapine (5 mg/kg) for a period of 20 days. Olanzapine treatment did not significantly change the average weight compared to vehicle treatment (Fig. 3A). Additionally, olanzapine treatment did not significantly change the average blood glucose compared to vehicle treatment (Fig. 3B).

We next looked at the effect of olanzapine treatment on insulin and leptin levels. Insulin and leptin levels in the treated group were not significantly different from vehicle group. In olanzapine-treated animals, insulin was 0.70 ± 0.10 ng/mL (range 0.43-1.03; n = 6) while insulin was 0.80 ± 0.10 ng/mL in vehicle-treated animals (range 0.49-1.33; n = 7; $P > 0.05$) (Fig. 3C). In olanzapine-treated animals, leptin was 732.0 ± 126.0 pg/mL (range 346.4-1256; n = 6) while leptin level was 763.2 ± 147.4 pg/mL in vehicle-treated animals (range 371.5-1360; n = 7; $P > 0.05$) (Fig. 3D).

Subchronic olanzapine treatment alters the regulation of excitatory inputs to DMV by olanzapine.

After 20 days of olanzapine treatment, recordings were made to investigate the effect of subchronic olanzapine treatment on the excitatory inputs to DMV neurons. DMV neurons in vehicle-treated animals displayed a similar response to acute olanzapine then in control animals. Bath application of olanzapine significantly increased the overall frequency of sEPSCs (Fig. 4A, 4B). In control conditions, the average sEPSC frequency was 3.00 ± 0.86 Hz (range 0.87-7.82 Hz). After 5 minutes of olanzapine infusion, the average sEPSC frequency was increased to 3.71 ± 0.92 Hz (range 0.88 - 8.36 Hz; n = 9; $P < 0.01$) (Fig. 4A, 4B). Additional analysis with Kolmogorov-Smirnov test revealed that 5 out of 9 cells had a significant increase in sEPSC frequency (Fig. 4B).

Bath application of olanzapine in olanzapine-treated animals resulted in a significant decrease of sEPSC frequency. In control conditions, the average sEPSC frequency was 1.87 ± 0.44 Hz (range 0.17-6.65 Hz). After 5 minutes of olanzapine infusion, the average sEPSC frequency was decreased to 1.23 ± 0.24 Hz (range 0.07 - 3.57 Hz; n = 17; $P < 0.01$) (Fig. 4A, 4B). Additional analysis with Kolmogorov-Smirnov test revealed that 10 out of 17 cells had a significant decrease in sEPSC frequency (Fig. 4B).

In summary, bath application of olanzapine resulted in a net increase of sEPSC frequency in control animals (53.54 ± 16.95 %; range 11.76 – 176.6 %; n = 9) and in vehicle-treated animals (32.02 ± 11.03 %; range 2.08 – 107.3 %; n = 9) (Fig. 4C). Co-application of TTX and olanzapine failed to increase mEPSC frequency (-6.05 ± 5.17 %; range -32.59 - 20.00 %; n = 10; $P < 0.001$) (Fig. 4C). Finally, bath application of olanzapine resulted in a net decrease of EPSC frequency in olanzapine-treated animals (-27.61 ± 5.27 %; range -57.41 – 10.60 %; n = 17; $P < 0.001$) (Fig. 4C).

These results indicate that subchronic administration of olanzapine for 20 days causes an altered response of DMV neurons to olanzapine, which indicates that neuronal changes occurred.

Subchronic olanzapine treatment changes some components of neuronal excitability of DMV neurons.

We then looked at the overall effect of subchronic olanzapine administration on the neuronal excitability of DMV neurons. The firing rate of APs in olanzapine-treated animals did not differ from vehicle-treated animals (Fig. 5A, 5B). Additionally, DMV neurons responded in a similar fashion to bath application of olanzapine (Fig. 5A, 5B). In vehicle-treated animals, the average AP frequency was 2.51 ±

0.46 Hz (range 0.77-4.11 Hz). Bath application of olanzapine significantly reduced the average AP frequency to 0.18 ± 0.10 Hz (range 0-0.67 Hz; n = 7; $P < 0.01$) (Fig. 5A, 5B). In olanzapine-treated animals, the average AP frequency was 3.39 ± 0.87 Hz (range 0.14-8.47 Hz). Bath application of olanzapine significantly reduced the average AP frequency to 0.38 ± 0.26 Hz (range 0-3.16 Hz; n = 12; $P < 0.01$) (Fig. 5A, 5B).

Interestingly, the RMP in olanzapine-treated animals (-42.73 ± 0.89 mV; range -46.82 - -36.96 mV; n = 12) was significantly more depolarized then compared to vehicle-treated animals (-45.92 ± 1.15 mV; range -50.51 - -42.30mV; n = 7; $P < 0.05$) (Fig. 5C). DMV neurons in both olanzapine-treated and vehicle-treated animals responded in a similar fashion to bath application of olanzapine (Fig. 5C).

In summary, bath application of olanzapine hyperpolarized DMV neurons to the same extent in controls conditions, in TTX, in vehicle-treated animals, and in olanzapine-treated animals (Fig. 5D). No significant difference was observed between the different conditions.

Finally, DMV neurons of olanzapine-treated animals had a lower input resistance (0.86 ± 0.07 GΩ (range 0.68-1.24 GΩ; n = 6) then in vehicle-treated animals 1.18 ± 0.12 GΩ (range 0.79-1.57 GΩ; n = 7; $P < 0.05$) (Fig. 5E). DMV neurons in both olanzapine-treated and vehicle-treated animals responded in a similar fashion to bath application of olanzapine (Fig. 5E).

Taken together, this indicates that some aspects of the neuronal excitability (i.e. RMP and input resistance) are altered in olanzapine-treated animals compared to vehicle-treated animals.

DISCUSSION

Our study provides novel information about olanzapine-dependent regulation of synaptic transmission in the DVC. Our data demonstrate that acute olanzapine application on DMV neurons has both a pre- and postsynaptic effect: olanzapine potentiates spontaneous excitatory inputs to DMV neurons in an AP-dependent manner and olanzapine hyperpolarizes DMV neurons in a AP-independent manner. Additionally, we show that subchronic olanzapine treatment (20 days) changes the olanzapine-dependent regulation of excitatory inputs: in olanzapine-treated animals, acute olanzapine administration reduces excitatory inputs to DMV neurons, which is opposite to the effect found in vehicle-treated animals. Finally, we show that the olanzapine-dependent hyperpolarization of DMV neurons is still present in olanzapine-treated animals. This demonstration of olanzapine-dependent regulation of DMV neurons suggests that olanzapine alters autonomic circuitry, possibly contributing to weight gain and hyperglycemia by decreasing the excitability of DMV neurons and reducing vagal output.

Technical considerations

We used female C57BL/6J mice to investigate both the acute and subchronic effect of olanzapine on DMV neurons. Female C57BL/6J mice are commonly used in studies looking at the link between atypical antipsychotics and metabolic dysregulation (Cope et al., 2007; 2005; Li et al., 2013). Female mice given atypical antipsychotics display similar weight gain as seen in human using these medications, which suggests that C57BL/6J female mice constitute a viable model for this study (Cope et al., 2005). However, in our study, we used a different route of administration then studies done with C57BL/6J female mice (e.g. peanut butter pills containing olanzapine): to better control for the dose received per animal, subcutaneous injections were performed daily, as is done with other rodent models used to study olanzapine-induced weight gain (Mann et al., 2013).

Subchronic injection of olanzapine (20 days at 5 mg/kg) failed to elicit weight gain or significant changes in metabolic characteristics (blood glucose, insulin, and leptin levels). This allowed us to investigate if neuronal changes occur before the onset of metabolic dysregulation. We hypothesized that early neuronal changes (i.e. prior to metabolic dysregulation) could contribute to metabolic dysregulation seen in chronically treated animals. However, to establish olanzapine-dependent neuronal changes during metabolic dysregulation will require additional investigation and could be the subject of future studies.

Olanzapine-dependent regulation of DMV neurons

Overall, olanzapine reduced the activity of DMV neurons, thus potentially reducing the vagal outflow to various subdiaphragmatic organs (Fig. 2, 5). Vagal activity plays a key role in diabetes: reduced vagal activity is strongly associated with type 2 diabetes in humans (Carnethon et al., 2003; Liao et al., 1995). The vagus nerve has direct connections to the liver, where it exerts tonic control over glucose production (Pocai et al., 2005). Concurrently, the vagal output also plays a role in energy homeostasis. Although controversial, the vagus is thought to innervate subcutaneous and abdominal fat, providing a direct control over leptin production (Giordano et al., 2006; Kreier et al., 2002). Additionally, hormonal modulation of the DVC by leptin and by melanocortin-3/4 directly reduces feeding through the vagal output, suggesting that the vagal outflow is a key regulator of satiety (Grill et al., 2002; Williams et al., 2000). By reducing the neuronal excitability of DMV neurons, olanzapine could reduce the vagal output, potentially perturbing both blood glucose regulation and energy homeostasis.

Specifically, olanzapine-dependent hyperpolarization of DMV neurons was present in both control animals and in olanzapine-treated animals (Fig. 5D). Also, TTX failed to block the hyperpolarization caused by olanzapine, suggesting that the effect is postsynaptic (Fig. 2B). Since olanzapine exhibits high binding affinity to various receptors (in particular $5HT_{2A}$ and to a minor extent dopamine D_2), it is likely that olanzapine exerts this hyperpolarization through one of these receptors. First-generation antipsychotics target mainly dopamine D_2 and do not show significant metabolic disturbances, suggesting that the dopamine system is not involved in the disturbance of metabolism (Allison et al., 1999). Interestingly, it has been reported that serotonin receptors are present in the DMV and participate in the regulation of the vagal output: postsynaptic $5HT_{2A}$ receptor activation depolarizes DMV neurons through reduction of K^+ conductance (Albert et al., 1996; Ballanyi and Kulik, 1998; Browning and Travagli, 1999; Hopwood and Trapp, 2005). Application of a $5HT_{2A}$ receptor antagonist, ketanserin, generates an outward current in some DMV neurons, suggesting that $5HT_{2A}$ is basally active in the DMV (Browning and Travagli, 1999). Olanzapine, as an inverse agonist for $5HT_{2A}$ receptor, could have a similar effect than ketanserin and reduce the basal activity of $5HT_{2A}$, hyperpolarizing DMV neurons by increasing K^+ conductance.

On the other hand, olanzapine-dependent regulation of spontaneous excitatory inputs to DMV neurons was altered in olanzapine-treated animals (Fig. 4C). This suggests that subchronic treatment

induces persistent neuronal changes that affect the response of excitatory inputs to olanzapine. Since TTX blocked olanzapine-dependent regulation of excitatory inputs, the effect is likely to be mediated by AP-dependent mechanisms (Fig. 1B). The brainstem slices used in our experiments contain intact NTS neurons that send both glutamatergic and GABAergic inputs to DMV neurons (Travagli and Rogers, 2001). Olanzapine could potentiate excitatory inputs to the DMV by either having a direct effect on NTS neurons, or through retrograde messengers that will affect NTS neurons. Clearly, future studies will be required to fully elucidate the nature of the mechanisms by which olanzapine reduces neuronal excitability of DMV neurons and potentiates spontaneous excitatory inputs to DMV neurons.

Together, we identified two distinct mechanisms by which olanzapine regulated neuronal transmission in the DMV. One mechanism (hyperpolarization of DMV neurons) is not altered by subchronic treatment while regulation of excitatory inputs to DMV neurons is altered (Fig. 6). Future studies will be required to fully elucidate the nature of the mechanisms by which olanzapine reduces neuronal excitability of DMV neurons.

In conclusion, our data strongly support the hypothesis that olanzapine (both acute and subchronic) disrupts normal neuronal transmission in the DVC and could be a pivotal factor in the development of autonomic imbalance ultimately leading to weight gain and hyperglycemia.

REFERENCES

Albert, A.P., Spyer, K.M., and Brooks, P.A. (1996). The effect of 5-HT and selective 5-HT receptor agonists and antagonists on rat dorsal vagal preganglionic neurones in vitro. Br. J. Pharmacol. *119*, 519–526.

Allison, D.B., Mentore, J.L., Heo, M., Chandler, L.P., Cappelleri, J.C., Infante, M.C., and Weiden, P.J. (1999). Antipsychotic-induced weight gain: a comprehensive research synthesis. Am J Psychiatry *156*, 1686–1696.

American Diabetes Association, American Psychiatric Association, American Association of Clinical Endocrinologists, North American Association for the Study of Obesity (2004). Consensus development conference on antipsychotic drugs and obesity and diabetes. pp. 267–272.

Ballanyi, K., and Kulik, A. (1998). Intracellular Ca2+ during metabolic activation of KATP channels in spontaneously active dorsal vagal neurons in medullary slices. Eur J Neurosci *10*, 2574–2585.

Bauer, S., Hay, M., Amilhon, B., Jean, A., and Moyse, E. (2005). In vivo neurogenesis in the dorsal vagal complex of the adult rat brainstem. Neuroscience *130*, 75–90.

Beasley, C.M., Tollefson, G., Tran, P., Satterlee, W., Sanger, T., and Hamilton, S. (1996). Olanzapine versus placebo and haloperidol: acute phase results of the North American double-blind olanzapine trial. Neuropsychopharmacology *14*, 111–123.

Berthoud, H.-R. (2002). Multiple neural systems controlling food intake and body weight. Neurosci Biobehav Rev *26*, 393–428.

Blouin, M., Tremblay, A., Jalbert, M.-E., Venables, H., Bouchard, R.-H., Roy, M.-A., and Alméras, N. (2012). Adiposity and Eating Behaviors in Patients Under Second Generation Antipsychotics. Obesity *16*, 1780–1787.

Browning, K.N., and Travagli, R.A. (1999). Characterization of the in vitro effects of 5-hydroxytryptamine (5-HT) on identified neurones of the rat dorsal motor nucleus of the vagus (DMV). Br. J. Pharmacol. *128*, 1307–1315.

Browning, K.N., and Travagli, R.A. (2011). Plasticity of vagal brainstem circuits in the control of gastrointestinal function. Autonomic Neuroscience: Basic and Clinical *161*, 6–13.

Bymaster, F.P., Calligaro, D.O., Falcone, J.F., Marsh, R.D., Moore, N.A., Tye, N.C., Seeman, P., and Wong, D.T. (1996). Radioreceptor binding profile of the atypical antipsychotic olanzapine. Neuropsychopharmacology *14*, 87–96.

Carnethon, M.R., Jacobs, D.R., Sidney, S., Liu, K., CARDIA study (2003). Influence of autonomic nervous system dysfunction on the development of type 2 diabetes: the CARDIA study. Diabetes Care *26*, 3035–3041.

Cavanaugh, D.J., Chesler, A.T., Jackson, A.C., Sigal, Y.M., Yamanaka, H., Grant, R., O'Donnell, D., Nicoll, R.A., Shah, N.M., Julius, D., et al. (2011). Trpv1 Reporter Mice Reveal Highly Restricted Brain Distribution and Functional Expression in Arteriolar Smooth Muscle Cells. Journal of Neuroscience *31*, 5067–5077.

Cooper, G.D., Pickavance, L.C., Wilding, J.P.H., Halford, J.C.G., and Goudie, A.J. (2005). A parametric analysis of olanzapine-induced weight gain in female rats. Psychopharmacology (Berl.) *181*, 80–89.

Cope, M.B., Jumbo-Lucioni, P., Walton, R.G., Kesterson, R.A., Allison, D.B., and Nagy, T.R. (2007). No

effect of dietary fat on short-term weight gain in mice treated with atypical antipsychotic drugs. Int J Obes Relat Metab Disord *31*, 1014–1022.

Cope, M.B., Nagy, T.R., Fernández, J.R., Geary, N., Casey, D.E., and Allison, D.B. (2005). Antipsychotic drug-induced weight gain: development of an animal model. Int J Obes Relat Metab Disord *29*, 607–614.

Cunningham, E.T., and Sawchenko, P.E. (1989). A circumscribed projection from the nucleus of the solitary tract to the nucleus ambiguus in the rat: anatomical evidence for somatostatin-28-immunoreactive interneurons subserving reflex control of esophageal motility. J. Neurosci. *9*, 1668–1682.

Davis, S.F., Williams, K.W., Xu, W., Glatzer, N.R., and Smith, B.N. (2003). Selective enhancement of synaptic inhibition by hypocretin (orexin) in rat vagal motor neurons: implications for autonomic regulation. Journal of Neuroscience *23*, 3844–3854.

Derbenev, A.V., Stuart, T.C., and Smith, B.N. (2004). Cannabinoids suppress synaptic input to neurones of the rat dorsal motor nucleus of the vagus nerve. J. Physiol. (Lond.).

Deutsch, J.A. (1985). The role of the stomach in eating. Am. J. Clin. Nutr. *42*, 1040–1043.

Fulton, B., and Goa, K.L. (1997). Olanzapine. A review of its pharmacological properties and therapeutic efficacy in the management of schizophrenia and related psychoses. Drugs *53*, 281–298.

Gao, H., Miyata, K., Bhaskaran, M.D., Derbenev, A.V., and Zsombok, A. (2012). Transient Receptor Potential Vanilloid Type 1-Dependent Regulation of Liver-Related Neurons in the Paraventricular Nucleus of the Hypothalamus Diminished in the Type 1 Diabetic Mouse. Diabetes *61*, 1381–1390.

Giordano, A., Song, C.K., Bowers, R.R., Ehlen, J.C., Frontini, A., Cinti, S., and Bartness, T.J. (2006). White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation. AJP: Regulatory, Integrative and Comparative Physiology *291*, R1243–R1255.

Glatzer, N.R., and Smith, B.N. (2005). Modulation of synaptic transmission in the rat nucleus of the solitary tract by endomorphin-1. J. Neurophysiol. *93*, 2530–2540.

Grill, H.J., Schwartz, M.W., Kaplan, J.M., Foxhall, J.S., Breininger, J., and Baskin, D.G. (2002). Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. Endocrinology *143*, 239–246.

Hopwood, S.E., and Trapp, S. (2005). TASK-like K+ channels mediate effects of 5-HT and extracellular pH in rat dorsal vagal neurones in vitro. J. Physiol. (Lond.) *568*, 145–154.

Kreier, F., Fliers, E., Voshol, P.J., Van Eden, C.G., Havekes, L.M., Kalsbeek, A., Van Heijningen, C.L., Sluiter, A.A., Mettenleiter, T.C., Romijn, J.A., et al. (2002). Selective parasympathetic innervation of subcutaneous and intra-abdominal fat — functional implications. J. Clin. Invest. *110*, 1243–1250.

Leucht, S., Pitschel-Walz, G., Abraham, D., and Kissling, W. (1999). Efficacy and extrapyramidal side-effects of the new antipsychotics olanzapine, quetiapine, risperidone, and sertindole compared to conventional antipsychotics and placebo. A meta-analysis of randomized controlled trials. Schizophr. Res. *35*, 51–68.

Li, X., Johnson, M.S., Smith, D.L., Jr, Li, Y., Kesterson, R.A., Allison, D.B., and Nagy, T.R. (2013). Effects of risperidone on energy balance in female C57BL/6J mice. Obesity n/a–n/a.

Liao, D., Cai, J., Brancati, F.L., Folsom, A., Barnes, R.W., Tyroler, H.A., and Heiss, G. (1995). Association of vagal tone with serum insulin, glucose, and diabetes mellitus--The ARIC Study. Diabetes

Res. Clin. Pract. *30*, 211–221.

Mann, S., Chintoh, A., Giacca, A., Fletcher, P., Nobrega, J., Hahn, M., and Remington, G. (2013). Pharmacology, Biochemistry and Behavior. Pharmacology, Biochemistry and Behavior *103*, 717–722.

Marino, J.S., Xu, Y., and Hill, J.W. (2011). Central insulin and leptin-mediated autonomic control of glucose homeostasis. Trends Endocrinol. Metab. *22*, 275–285.

Moore, N.A., Tye, N.C., Axton, M.S., and Risius, F.C. (1992). The behavioral pharmacology of olanzapine, a novel "atypical" antipsychotic agent. J. Pharmacol. Exp. Ther. *262*, 545–551.

Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S., and Schwartz, M.W. (2006). Central nervous system control of food intake and body weight. Nature *443*, 289–295.

Mussa, B.M., and Verberne, A.J.M. (2013). The dorsal motor nucleus of the vagus and regulation of pancreatic secretory function. Experimental Physiology *98*, 25–37.

Pocai, A., Obici, S., Schwartz, G.J., and Rossetti, L. (2005). A brain-liver circuit regulates glucose homeostasis. Cell Metabolism *1*, 53–61.

Ratzoni, G., Gothelf, D., Brand-Gothelf, A., Reidman, J., Kikinzon, L., Gal, G., Phillip, M., Apter, A., and Weizman, R. (2002). Weight gain associated with olanzapine and risperidone in adolescent patients: a comparative prospective study. J Am Acad Child Adolesc Psychiatry *41*, 337–343.

Roussin, A.T., D'Agostino, A.E., Fooden, A.M., Victor, J.D., and Di Lorenzo, P.M. (2012). Taste Coding in the Nucleus of the Solitary Tract of the Awake, Freely Licking Rat. Journal of Neuroscience *32*, 10494–10506.

Sandoval, D., Cota, D., and Seeley, R.J. (2008). The Integrative Role of CNS Fuel-Sensing Mechanisms in Energy Balance and Glucose Regulation. Annu. Rev. Physiol. *70*, 513–535.

Shoudai, K., Peters, J.H., McDougall, S.J., Fawley, J.A., and Andresen, M.C. (2010). Thermally Active TRPV1 Tonically Drives Central Spontaneous Glutamate Release. Journal of Neuroscience *30*, 14470–14475.

Travagli, R.A., and Rogers, R.C. (2001). V. Fast and slow extrinsic modulation of dorsal vagal complex circuits. Am. J. Physiol. Gastrointest. Liver Physiol. *281*, G595–G601.

Travagli, R.A., Gillis, R.A., Rossiter, C.D., and Vicini, S. (1991). Glutamate and GABA-mediated synaptic currents in neurons of the rat dorsal motor nucleus of the vagus. Am. J. Physiol. *260*, G531–G536.

Weston-Green, K., Huang, X.-F., and Deng, C. (2012). Alterations to Melanocortinergic, GABAergic and Cannabinoid Neurotransmission Associated with Olanzapine-Induced Weight Gain. PLoS ONE *7*, e33548.

Weston-Green, K., Huang, X.-F., Han, M., and Deng, C. (2008). The effects of antipsychotics on the density of cannabinoid receptors in the dorsal vagal complex of rats: implications for olanzapine-induced weight gain. Int. J. Neuropsychopharm. *11*.

Williams, D.L., Kaplan, J.M., and Grill, H.J. (2000). The role of the dorsal vagal complex and the vagus nerve in feeding effects of melanocortin-3/4 receptor stimulation. Endocrinology *141*, 1332–1337.

Williams, K.W., Zsombok, A., and Smith, B.N. (2006). Rapid Inhibition of Neurons in the Dorsal Motor Nucleus of the Vagus by Leptin. Endocrinology *148*, 1868–1881.

Zhang, W., and Bymaster, F.P. (1999). The in vivo effects of olanzapine and other antipsychotic agents on

receptor occupancy and antagonism of dopamine D1, D2, D3, 5HT2A and muscarinic receptors. Psychopharmacology (Berl.) *141*, 267–278.

Zhang, W., Zhang, C., Fritze, D., Chai, B., Li, J., and Mulholland, M.W. (2013). Modulation of food intake by mTOR signalling in the dorsal motor nucleus of the vagus in male rats: focus on ghrelin and nesfatin-1. Experimental Physiology *98*, 1696–1704.

Zsombok, A., and Smith, B.N. (2009). Plasticity of central autonomic neural circuits in diabetes. Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease *1792*, 423–431.

Zsombok, A., Bhaskaran, M.D., Gao, H., Derbenev, A.V., and Smith, B.N. (2011a). Functional plasticity of central TRPV1 receptors in brainstem dorsal vagal complex circuits of streptozotocin-treated hyperglycemic mice. Journal of Neuroscience *31*, 14024–14031.

Zsombok, A., Gao, H., Miyata, K., Issa, A., and Derbenev, A.V. (2011b). Immunohistochemical localization of transient receptor potential vanilloid type 1 and insulin receptor substrate 2 and their co-localization with liver-related neurons in the hypothalamus and brainstem. Brain Research *1398*, 30–39.

FIGURES

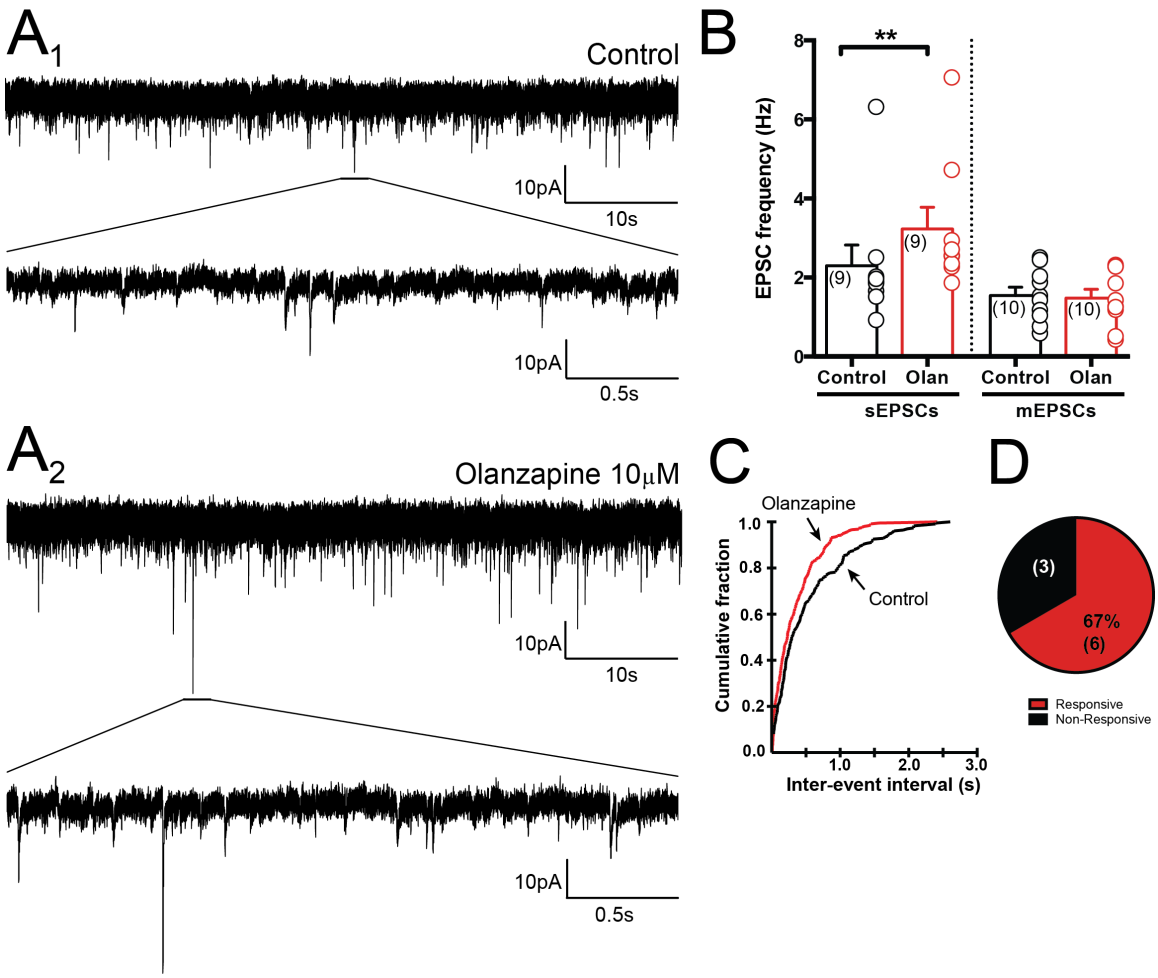

Figure 1. Olanzapine potentiates excitatory inputs to DMV neurons in a calcium-dependent manner. **A**. Continuous recording of sEPSCs in control conditions ($\mathbf{A_1}$) and after bath application of olanzapine 10 μM ($\mathbf{A_2}$). **B**. Combined data showing the effect of olanzapine on EPSC frequency. **C**. Cumulative event probability plots of inter-event interval distribution in recordings from DMV neurons. **D**. Potentiation of DMV neurons by acute application of olanzapine. Replication numbers are in parenthesis. **Significance ($p < 0.01$).

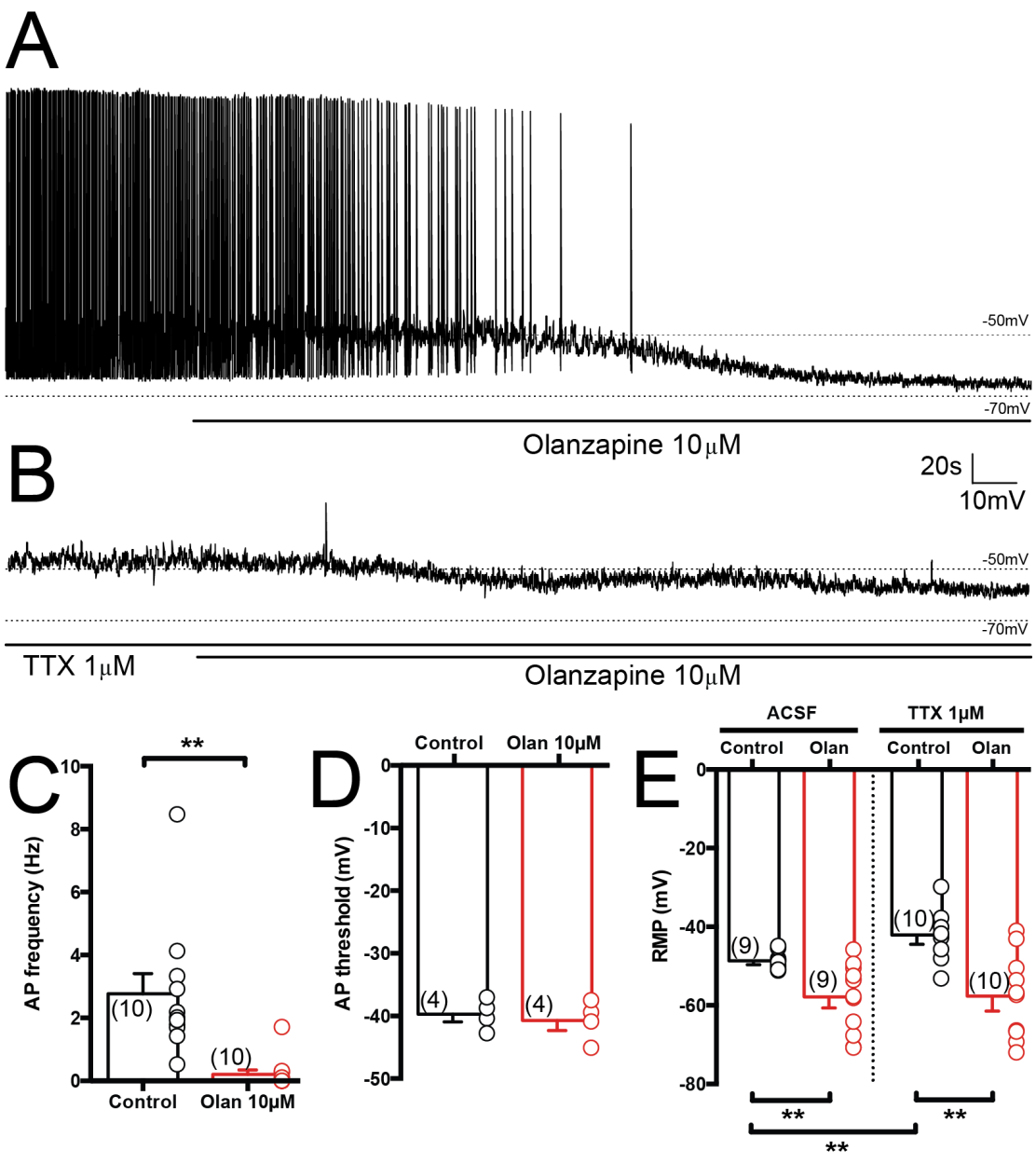

Figure 2. Olanzapine reduces the neuronal excitability of DMV neurons in a calcium-independent fashion. **A**. Continuous recording at 0 pA in control conditions showing that olanzapine suppresses APs and hyperpolarizes DMV neurons. **B**. Continuous recording at 0 pA in TTX 1 μM showing that olanzapine suppresses APs and hyperpolarizes DMV neurons. **C-E**. Combined data showing the effect of olanzapine on AP frequency (**C**), AP threshold (**D**), and RMP (**E**). Replication numbers are in parenthesis. **Significance ($P < 0.01$).

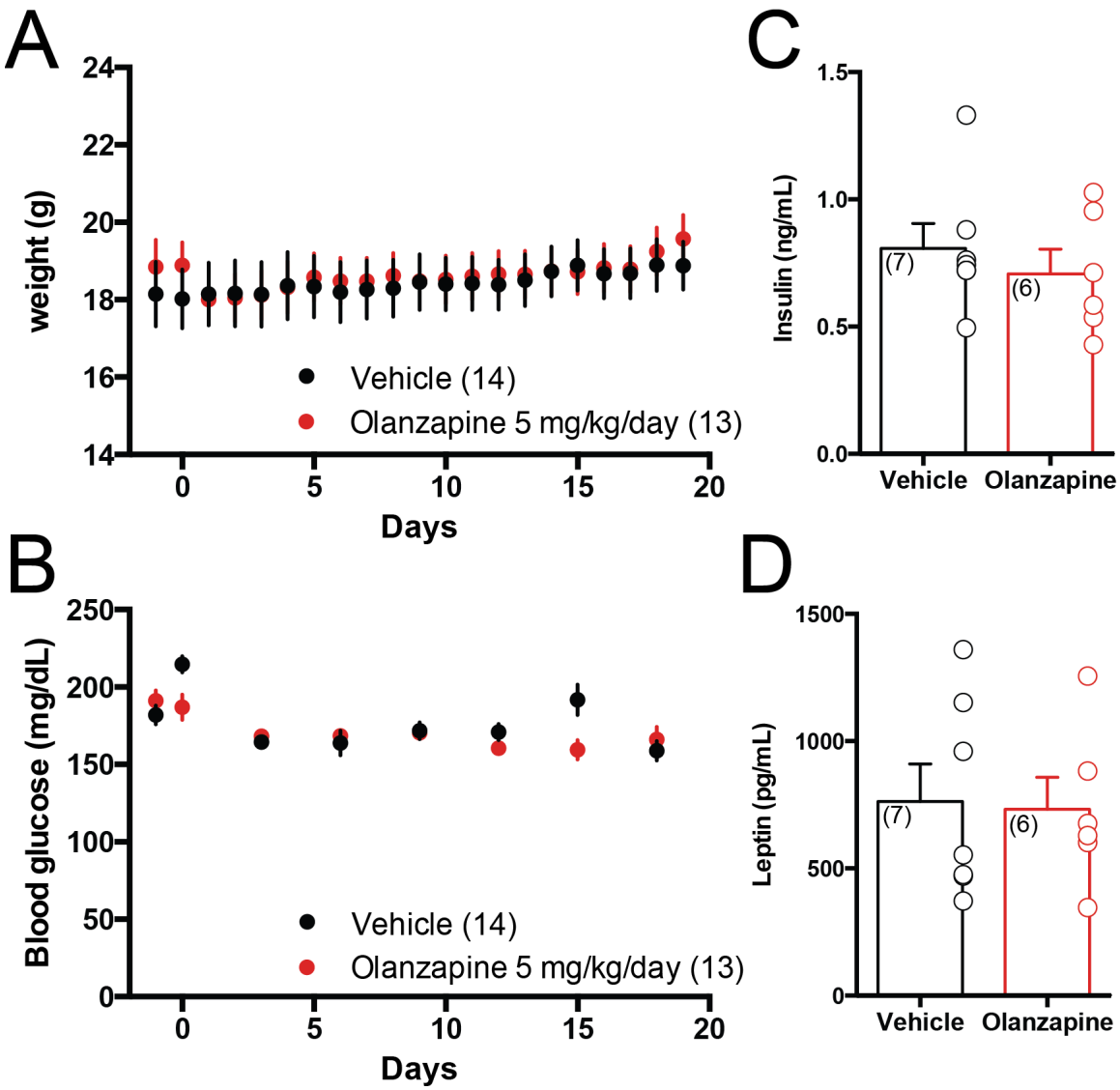

Figure 3. Subchronic olanzapine treatment does not affect metabolic characteristics **A-B**. Combined data showing the changes in body weight (**A**) and blood glucose (**B**) over the 20-days olanzapine treatment. **C-D**. Combined data showing the effect of olanzapine treatment on insulin (**C**), and leptin (**D**). Replication numbers are in parenthesis.

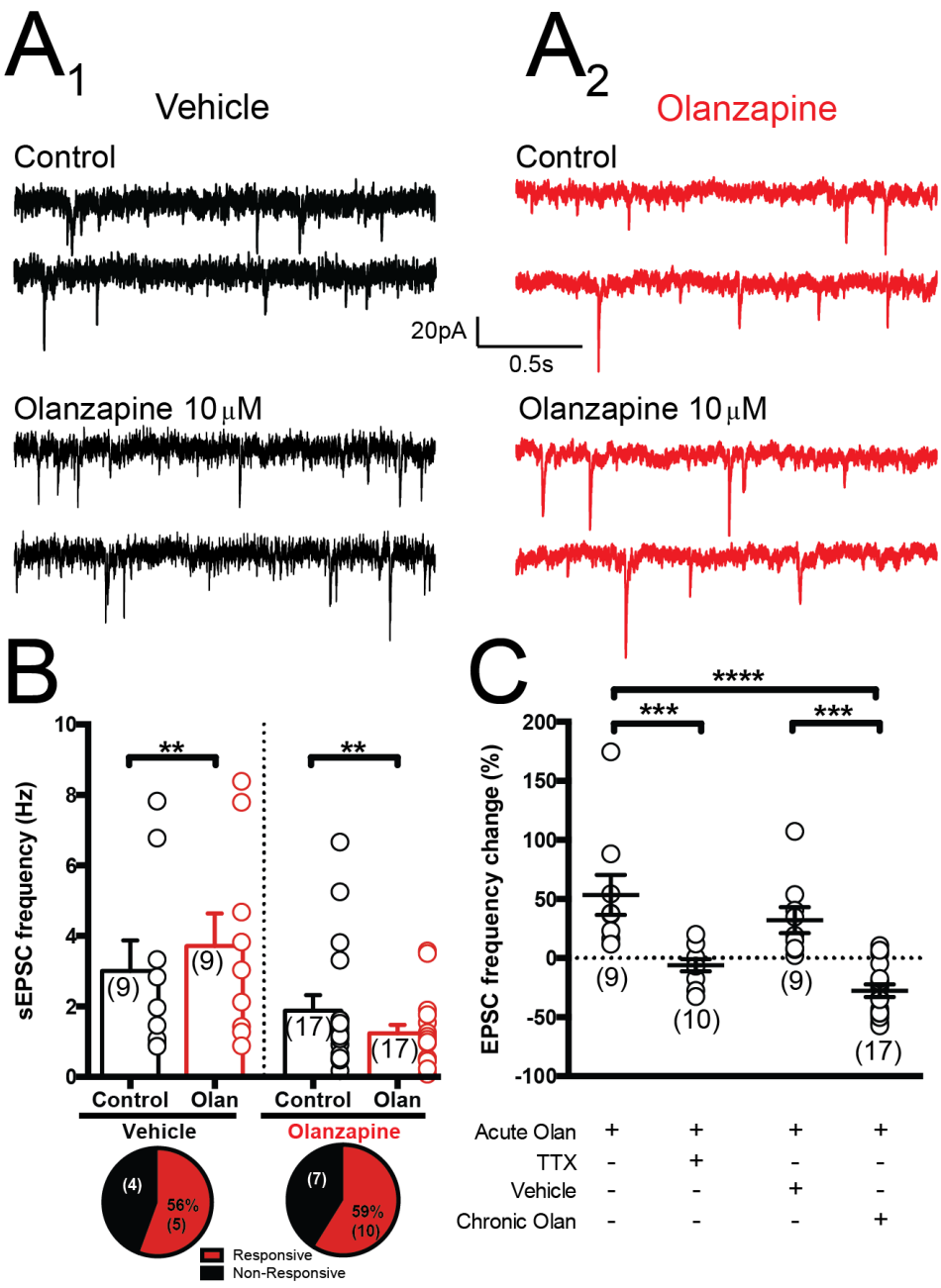

Figure 4. Subchronic olanzapine treatment alters the regulation of excitatory inputs to DMV by olanzapine. **A**. Recording of sEPSCs in vehicle-treated animals ($\mathbf{A_1}$) and in olanzapine-treated animals ($\mathbf{A_2}$). **B**. Combined data showing the differential effect of olanzapine on sEPSC frequency in vehicle-treated animals and in olanzapine-treated animals. **C**. Summary data showing differential responses to acute olanzapine application in different conditions. Replication numbers are in parenthesis. **Significance ($p < 0.01$). ***Significance ($p < 0.001$). ****Significance ($p < 0.0001$).

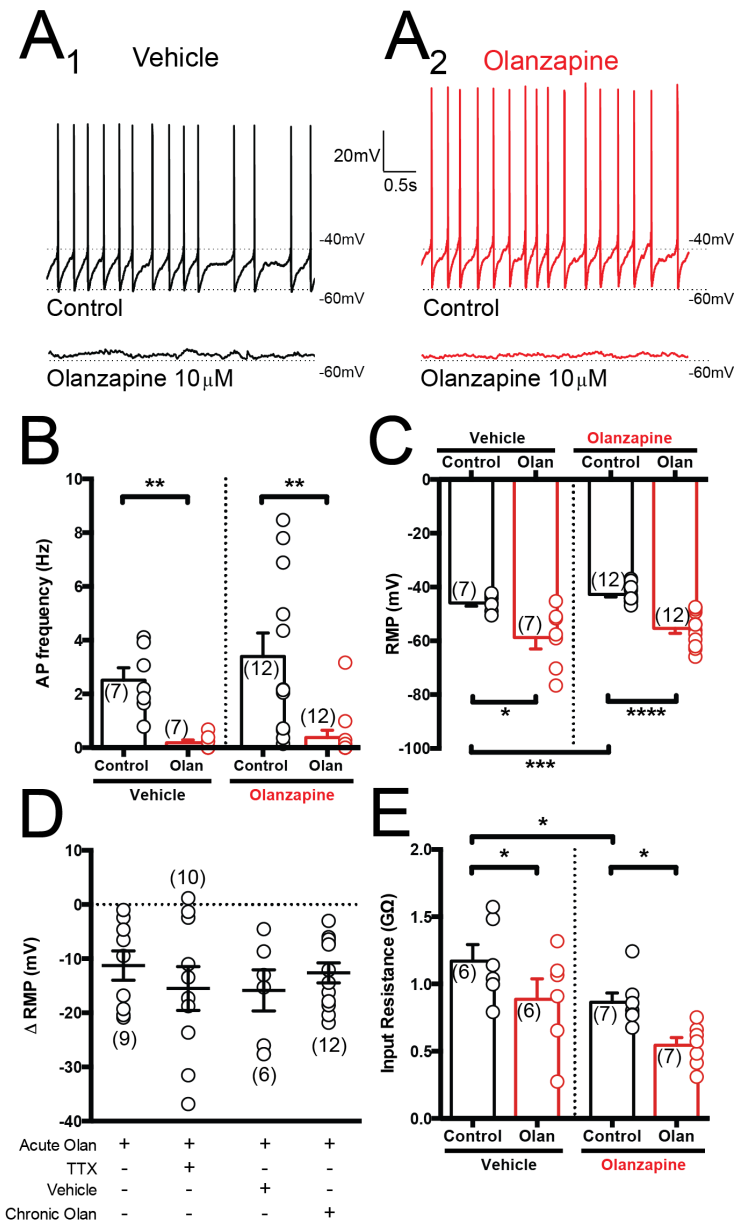

Figure 5. Subchronic olanzapine treatment changes some components of neuronal excitability of DMV neurons. **A**. Recording at 0 pA in vehicle-treated animals (**A$_1$**) and in olanzapine-treated animals (**A$_2$**). **B-C**. Combined data showing the effect of olanzapine on AP frequency (**B**) and RMP (**C**) in vehicle-treated animals and in olanzapine-treated animals. **D**. Summary data showing similar olanzapine-induced hyperpolarization in different conditions. **E**. Combined data showing the effect of olanzapine on input resistance in vehicle-treated animals and in olanzapine-treated animals. Replication numbers are in parenthesis. *Significance (P < 0.05). **Significance (p < 0.01). ***Significance (p < 0.001). ****Significance (p < 0.0001).

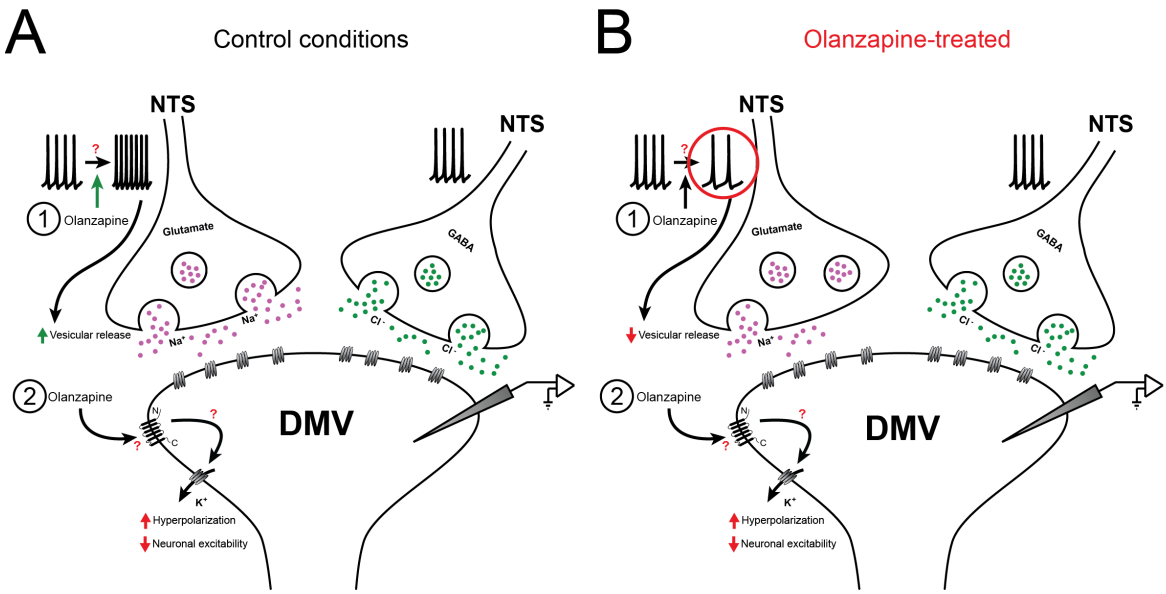

Figure 6. Subchronic olanzapine treatment alters olanzapine-mediated presynaptic effect on excitatory inputs to DMV neurons. **A**. Schematic showing two distinct mechanisms (presynaptic (1) and postsynaptic (2)) by which olanzapine acts on DMV neurons. **B**. The presynaptic effect is altered in olanzapine-treated animals while postsynaptic effect remains identical.

BIOGRAPHY

Imran Anwar, raised in Lausanne, Switzerland, graduated from the Gymnase de Beaulieu in 2008. He entered Tulane University in 2009, and completed his Bachelor of Science *summa cum laude* in Neuroscience and his Bachelor of Arts *cum laude* in Philosophy in May 2013.